Synthetic Organophosphate toxicity leading to Neurodegenerative Disease: Assessment through Biological, Neuronal and Molecular Parameters

SYNOPSIS

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1.0 INTRODUCTION

In the race of technology, we have come so far that the side effects of advancement have started showing up. By far, we must have mastered our physical deformities but our mental health is degrading gradually. Conquering the physical world is worthless until and unless we own a sound mind. Unhealthy eating habits, increasing population demands over agricultural yields, mishandling and misapplication of pesticides, etc. are some common and overlooked problems that directly and indirectly affect our normal living (Zaganas *et al*., 2013). One of the most severe outcomes of these abuses is neurodegeneration (Palop *et al*., 2010). It is the progressive loss of structural as well as functional properties of neurons, leading to its dysfunction and ultimate degeneration of neurons. Various neurodegenerative diseases include Alzheimer's, amyotrophic lateral sclerosis, Parkinson's and Huntington's (Dimitriadi *et al*., 2010). Among these, Alzheimer's disease (AD) is the most common cause of dementia in the elderly, accounting for 60–70% of the cases of progressive cognitive impairment (Francis *et al*., 1999). The cause of Alzheimer's disease is poorly understood but studies suggests that most affected neurons includes cholinergic and glutamatergic neurons in the hippocampus and the neocortex regions of brain (Francis *et al*., 1999; Leung *et al*., 2008; Wang *et al.,* 2010). Clinically, the disease is characterized by the presence of senile plaques (SPs) and neurofibrillary tangles (NFTs) in the brain of affected individuals. The major constituents of SPs are toxic species of β-amyloid (Aβ) peptide, whereas NFTs contain hyper-phosphorylated and aggregated forms of the microtubule-associated protein tau (MAPT) (Dimitriadi *et al*., 2010). About 70% of the risk for development of AD is believed to be genetic, involving mutation in APP (Amyloid precursor protein), PSEN1 (Presenilin 1), PSEN2 (Presenilin 2) and APOE ε4 (Yankner *et al*., 1996; Palop *et al.,* 2011).

The role of environmental factors and their genetic susceptibility in the etiology of Alzheimer's disease have been reviewed in recent decades. It has been found that insecticides are the predominant neurotoxic class of pesticides, among which organophosphates (OPs) are most commonly used insecticides. These OPs are widely used in agriculture, home, garden and public health programs to target a number of pests

like cockroaches, grubs, flies, termites, fire ants, lice, etc. and mosquitoes as well due to their potency to inhibit Acetylcholine esterase (AChE) (Leung *et al.,* 2008; Jadhav *et al.,* 2009; Meyer *et al.,* 2014). Therefore, OPs are more promptly associated with the risk of developing Alzheimer's disease in exposed population (Thanyet *et al.,* 2013). Human exposure to organophosphate including monocrotophos, chlorpyrifos and dichlorvos are extensively reported due to their wide applications and presence as a contaminant in the dietary and food products (Yadav *et al*., 2016). In the present study, preliminary screening is required to select the most affecting pesticide, from the commonly used OPs such as omethoate, disulfoton, trichlorfon, azinphos-methyl and turbufos. Screening will be done by studying the effect of pesticides on various biological parameters, change in these indicating effect on neurons by any environmental toxicity (Tejeda *et al*., 2016).

To evaluate the proposed hypothesis, a model organism *Caenorhabditis elegans* will be used. Due to the cellular complexity and the conservation of disease pathways between *C. elegans* and higher organisms, the nematode worm has developed into an important model for toxicological research (Kaletta *et al*., 2006). Various characteristic features of this free living, soil nematode worm, making it a suitable model organism includes:

- Small, transparent body of ~1 mm in length for easy *in vivo* visualization of cells and organs (Boyd *et al*., 2010)
- Short generation time of 3-4 days and a total life span of ~3 weeks with high reproducible rate (Brenner, 1947).
- Fully sequenced genome with 42% of ortholog genes to human related diseases (Dimitriadi *et al*., 2010).
- Conserved fundamental biology of neurons with humans.

C. elegans have a simple nervous system of 302 neurons in the adult hermaphrodite, in which each neuron has a unique position and identity that is reproducible from animal to animal (White *et al*., 1986;

Dimitriadi *et al*., 2010). Therefore, *C. elegans* provide strong insight into the mechanism of neurodegeneration across species (Wolozin *et al*., 2011).

Our study focuses on the effect of selected OPs, using *C. elegans* as model organism at three levels i.e. biological, neuronal and molecular parameters. The change in the biological parameter incur adverse effect at the neuronal and molecular parameter. In the biological parameters, various behavioral and developmental changes will be analyzed under phase contrast microscope. The neuron level toxicity will be identified by visualizing the degeneration of Cholinergic and Glutamatergic neurons under dissecting fluorescence microscope. The gene level toxicity will be analyzed by gene expression of the human homologs i.e. APP-related gene, apl-1, a tau homolog, ptl-1, and presenilin homologs, such as sel-12 and hop-1 gene in *C. elegans* after pesticide exposure (Alexander *et al*., 2014). Gene expression analysis will be done by two step reverse transcription polymerase chain reaction (RT-PCR) followed by agarose gel electrophoresis.

These neurodegenerative diseases not only affect individual's life but also becomes a burden on society and caretakers. By far, no cure has been found that helps in long run. Consequently, our study would help in recognizing environmental agent that we are encountering in our daily life directly or indirectly, unaware of their hazardous effects upon us. Limiting the use of such pesticides can help in getting heathier life with sound mind as well.

2.0 REVIEW OF LITERATURE

In 1907, a German physician Dr. Alois Alzheimer first described "a peculiar disease" in a case of Auguste D., a patient who had profound memory loss, unfounded suspicions about her family, and other worsening psychological changes. Three year later in 1910, Emil Kraepelin, a German psychiatrist who worked with Dr. Alois Alzheimer, first named the disease as "Alzheimer's Disease" in the eighth edition of his book "Psychiatrie"

(http://www.alz.org/research/science/major_milestones_in_alzheimers.asp). In 1973, the pioneer work done on isolation, culture and use *C. elegans* as a model organism was done by Sydney Brenner. He isolated many mutants of the worm and mapped them. In 1984, Researcher George Glenner and Cai'ne Wong reported identification of "a novel cerebrovascular amyloid protein," known as beta-amyloid — the chief component of Alzheimer's brain plaques and a prime suspect in triggering nerve cell damage. In 1986, White, *et al.* described the structure and the connectivity of the nervous system of the nematode. T. Roy Fukuto in 1990 addressed the mechanism of inhibition of acetylcholinesterase by organophosphorus and carbamate esters, focusing on structural requirements necessary for anticholinesterase activity. In 1994, the first World Alzheimer's Day (WAD) was launched on September 21 by Alzheimer's Disease International, the Umbrella Organization of Alzheimer's associations. In 1996, Bruce A. Yankner, described the mechanisms of Neuronal Degeneration in Alzheimer's Disease suggesting that there are four different genes that confer susceptibility to AD which were the amyloid precursor protein (APP), apolipoprotein E (ApoE), and two novel seven transmembrane domain proteins PSEN1 and PSEN2. A review process was done by Francis *et al.*, in 1999 that suggested the cholinergic hypothesis of AD. He examined the existing scientific applicability of the original cholinergic hypothesis of Alzheimer's disease by describing the biochemical and histopathological changes of neurotransmitter markers. In further discoveries, Zhu *et al.*, in 2001 found a major cellular stress response protein, c-Jun N-terminal kinase/Stress activated protein kinase (JNK/SAPK) induced by oxidative stress, playing an important role in Alzheimer disease in susceptible neurons facing the dilemma of proliferation or death. Hardy, *et al* in 2002 detailed the Amyloid Hypothesis of Alzheimer's Disease. This provided progress and solutions to the problems on the road to therapeutics. In 2002, a reusable software components for model organism system databases was developed by Stein *et al.* They described the Generic Genome Browser (GBrowse), a Web-based application for displaying genomic annotations and other features. Szewczyk *et al*., in 2003 gave a Methodology article for chemically defining culturing medium for *Caenorhabditis elegans*. Early in 2004, Dengg and Meel used *Caenorhabditis elegans* as model

system for rapid toxicity assessment of pharmaceutical compounds. In their study, they used compounds like BIBU1361, BIBX1382, and BIBU1476 to assess endpoints like lethality, stress response and β-Galactosidase expression. Freya Kamel and Jane A. Hoppin in 2004 provided insight about association of pesticide exposure with neurologic dysfunction and disease. Takeshi Kihara and Shun Shimohama in 2004 detailed about Alzheimer's disease and acetylcholine receptors. Cholinergic abnormalities, alongside senile plaques, neurofibrillary tangles, and extensive neuronal loss, the major characteristics in Alzheimer's disease (AD) were well descried. In 2006, Theresa Stiernagle precisely described the maintenance of *C. elegans* in the laboratory. Starting from the acquisition of worm strains from the Caenorhabditis Genetics Center (CGC), to methods of culture and transfer, decontamination of stocks, synchronizing and staging cultures, and procedures for freezing worms for long-term storage and for recovering them from the frozen state are well described in the paper. In 2006, Titus Kaletta and Michael O. Hengartner proposed *C. elegans* to study a much larger variety of biological processes including apoptosis, cell signaling, cell cycle, cell polarity, gene regulation, metabolism, ageing and sex determination. Many key discoveries, both in basic biology and medically relevant areas, were first made in the worm. In 2007, Miguel *et al.,* showed a review on Occupational risk factors in Alzheimer's disease assessing the quality of published epidemiological studies. Leung *et al.,* in 2008 detailed *Caenorhabditis elegans* as An Emerging Model in Biomedical and Environmental Toxicology. They described some of the research that has been carried out in the areas of neurotoxicology, genetic toxicology, and environmental toxicology, as well as high-throughput experiments with *C. elegans* including genome-wide screening for molecular targets of toxicity and rapid toxicity assessment for new chemicals. Rajini *et al.,* in 2008 showed a comparative study on the relationship between various toxicological endpoints in *Caenorhabditis elegans* exposed to Organophosphorus Insecticides. They tested the toxicity of 10 organophophorus (OP) insecticides: acephate, dimethoate, dichlorvos, dicrotophos, monocrotophos, methami- dophos, phosphamidon, omethoate, phosdrin, and trichlorfon to evaluate lethality, movement, and acetylcholinesterase (AChE) activity as the endpoints after a 4-hr- exposure period. In 2009, Kisan B. Jadhav and

Padmanabhan Sharda Rajini Evaluated Sublethal Effects of Dichlorvos upon *Caenorhabditis elegans* Based on a Set of End Points of Toxicity namely, stress gene expression (hsp16), feeding, and acetylcholinesterase (AChE) activity. Corbel *et al.,* in 2009 used toxicological, biochemical and electrophysiological techniques, and showed that DEET is not simply a behaviour-modifying chemical but that it also inhibits cholinesterase activity, in both insect and mammalian neuronal preparations. In 2009, Lars Bertram and Rudolph E. Tanzi conducted Genome-wide association studies in Alzheimer's disease for the identification of novel complex disease genes. In 2010, Maria Dimitriadi and Anne C. Hart explored Neurodegenerative disorders insights from the nematode *Caenorhabditis elegans*. An overview of the clinical and genetic features of AD was given by Bekris *et al.,* in 2010. In 2010, Jorge J. Palop and Lennart Mucke researched Amyloid-β induced neuronal dysfunction in Alzheimer's Disease from synapses toward neural networks. Wang *et al.,* in 2010 did the Functional Genomics of Brain Aging and Alzheimer's Disease, focusing on selective neuronal vulnerability. Wolozin *et al*. in 2011 did modeling of neurodegeneration in *C. elegans* showing various disease related genes and their roles in the worm. By using High-throughput Screening and Biosensing with fluorescent *C. elegans* Strains, Leung *et al.,* in 2011 came out with a powerful approach for identifying chemical modulators of biological processes. Kamboh *et al.* Roh *et al.,* in 2011 showed that Cyp35a2 gene expression is involved in toxicity of fenitrothion in the soil nematode *Caenorhabditis elegans* by analysis various endpoints. Kratsios *et al*., in 2012 coordinated regulation of cholinergic motor neuron traits through a conserved terminal selector gene. McColl *et al.,* in 2012, provided *C. elegans* model of full length $Aß_{1-42}$ expression that was adopted for use in screens to rapidly identification and assessment in development of potential therapeutics and studied underlying toxic mechanism(s) of Aß. Anbalagan *et al.,* in 2012 used transgenic GFP reporter strains of the nematode *Caenorhabditis elegans* to investigate the patterns of stress responses induced by pesticides and by organic extracts from agricultural soils. In 2012 Kamboh *et al.,* did Genome-wide association study of Alzheimer's disease, and examined in detail the implicated nine new regions with Alzheimer's disease (AD) risk. Boyd *et al.,* in 2012 explored *Caenorhabditis elegans* as a model in

developmental toxicology with latest techniques. Colovic *et al.,* 2013 provided an overview of toxicology and pharmacology of reversible and irreversible acetylcholinesterase inactivating compounds. Dean Meyer & Phillip L. Williams preformed toxicity testing of neurotoxic pesticides chlorpyrifos and diazinon in *Caenorhabditis elegans* in 2014 and measured endpoints including changes in locomotion, feeding behavior, brood size, growth, life span, and cell death. Alexander *et al.,* in 2014 explored *Caenorhabditis elegans* as a model to study Alzheimer's disease and other neurodegenerative diseases. Yegambaram *et al.,* provided an overview of in vitro, animal and epidemiological studies on the etiology of AD, highlighting available data supportive of the long hypothesized link between toxic environmental exposures and development of AD pathology in 2015. Neurotoxicity of two related organophosphates on *Caenorhabditis elegans* was well tested by Collin m. Brown in 2016. A systematic review and meta-analysis was done by Yan *et al.,* to correlated Pesticide exposure and risk of Alzheimer's disease in 2016. The Epigenetic Aspect of Organophosphates Induced Alzheimer's Disease was explored by Sunishtha *et al.,* in 2016. Lesly Tejeda-Benitez and Jesus Olivero-Verbel in 2016 illuminated very precisely a biological model, *Caenorhabditis elegans* for research in toxicology through various endpoints. Mantzavinosa *et al.,* in 2017 described various Biomarkers for Alzheimer's Disease Diagnosis. By reviewing all such related studies in neurodegeneration, toxicology, *C. elegans* and molecular techniques, we found that work is been carried forward in decoding the disease but many more findings are still to be discovered. Various mechanism of onset of the disease are still unclear. To counter such unsolved questions, we need to work in the direction of prevention first. Our study focuses to find such harmful pesticides that have not been reviewed and analyzed over prescribed parameters. Therefore, we have formulated our research in direction of public health to ensure better living.

3.0 OBJECTIVES

- *In vivo* assessment of change in biological parameters in *C. elegans* due to synthetic organophosphates (OPs) toxicity.
- *In vivo* assessment of synthetic OPs toxicity on degeneration of Cholinergic, Pyramidal and Glutamatergic neurons in *C. elegans*.
- To study the effect of OPs toxicity on the gene expression of human ortholog neurodegenerative genes in *C. elegans*.

4.0 MATERIALS AND METHODS

4.1 MATERIALS

For all the toxicity assays, the wild-type N2 strain of *Caenorhabditis elegans* will be obtained from the Caenorhabditis Genetics Center (CGC) (University of Minneapolis, MN, USA). *C. elegans* cultures will be fed on the lawn of OP50 strain of *E. coli* which will also be procured from CGC (Boyd *et al.*, 2012).

4.2 METHODS

4.2.1 Worm Culture and pesticide Exposures

Culturing of *C. elegans* will be done on nematode growth medium (NGM) at 20°C for 3 days, preseeded with a lawn of *E. coli* OP50 (Leung *et al*., 2010). The Age-synchronized populations of N2 worms will be obtained by the collection and culturing of eggs laid through adult worm (Rajini *et al*., 2008). Concentrated pallet of *E. coli* OP50 will be obtained by incubating the culture for 24 hrs. at 37ºC, which will be then stored at 4°C for several weeks as stock (Stiernagle *et al.*, 2006). L1 and L2 stage of *C. elegans* will be frozen and stored indefinitely in liquid nitrogen (−80°C) by addition of glycerol to the freezing media (Brenner, 1974).

Treatment of the pesticides to the worm will be given in two ways. Long term exposure will be given on NGM plates preseeded with *E. coli* OP50 with the desired concentration of the pesticide using the methodology of Meyer *et al.* 2014. The acute exposure will be given by incubating the adult worms in the K medium containing the toxicant at several concentrations (Zhuang *et al*. 2014; Tejeda *et al*., 2016).

Further various bioassays to assess the effects of a toxicant on *C. elegans* will be carried out at three levels i.e. at biological, neuronal and molecular parameters.

4.3 BIOLOGICAL PARAMETERS

For the present study, biological parameters has been divided into two categories as behavioral and developmental endpoints.

4.3.1 Behavioral Endpoints

Behavioral changes will be evaluated by analyzing the effect on locomotion & paralysis, foraging & learning and reproductive behavior of *C. elegans* after the exposure of pesticides.

4.3.1.1 Locomotion and Paralysis

Effects on the locomotion of nematodes have been linked to a deterioration of the neural network which will be evaluated by head thrash, body bend frequency, and basic movements. Alternatively, paralysis will be determined by counting the number of immobile worms, usually registering a response when touched by metal wire (Jadhav *et al.*, 2009; Roh *et al.*, 2011; Tejeda *et al.*, 2016).

4.3.1.2 Foraging and Learning

The feeding and foraging behavior in *C. elegans* will be studied by keeping the treated nematodes in the center of the NGM plate, having holes at equidistance from the center filled with bacterial food suspension (*E. coli* OP50) and toxic solution. (Tejeda *et al.*, 2016).

4.3.1.3 Reproduction

After placing exposed adult or L4 worms onto fresh plates, the number of offspring at all stages will be counted and compared with a control group (Leelaja and Rajini 2013; Tejeda *et al*., 2016).

4.3.2 Developmental Endpoints

Developmental endpoints will be evaluated by analyzing the effect on lethality, growth and lifespan of *C. elegans* after pesticide exposure.

4.3.2.1 Lethality

Pesticide exposure at different concentration will be given to adult worm at 20ºC for 24 hrs. Then the number of live and dead worms will be counted through visual inspection using dissecting microscope (Zhuang *et al*. 2014; Tejeda *et al.,* 2016).

4.3.2.2 Growth

The effect of a toxicant in the development of the nematode will be evaluated by measuring the body length of synchronous worms before and after exposure. The body length of the worms will be measured in light microscope with 10X magnification and with image analysis software, such as Image-Pro ® Express, ImageJ, or Fiji (Boyd *et al*., 2010; Meyer *et al*., 2010; Roh *et al.,* 2011). The immobilization of the worms will be achieved by using sodium azide for better observations (Turner *et al*., 2013).

4.3.2.3 Lifespan

Healthy worms at the L4 larval stage will be exposed to the toxic agent, for about 24 hrs. and then placing them on fresh NGM plates with *E. coli* OP50. Likewise the worms will be transferred to new plates every 3 days. The number of survivors will be recorded every day until all animals die. The survival rate will be calculated by dividing the number of live nematodes by the total number of nematodes (Zhuang *et al*. 2014; Tejeda *et al.,* 2016).

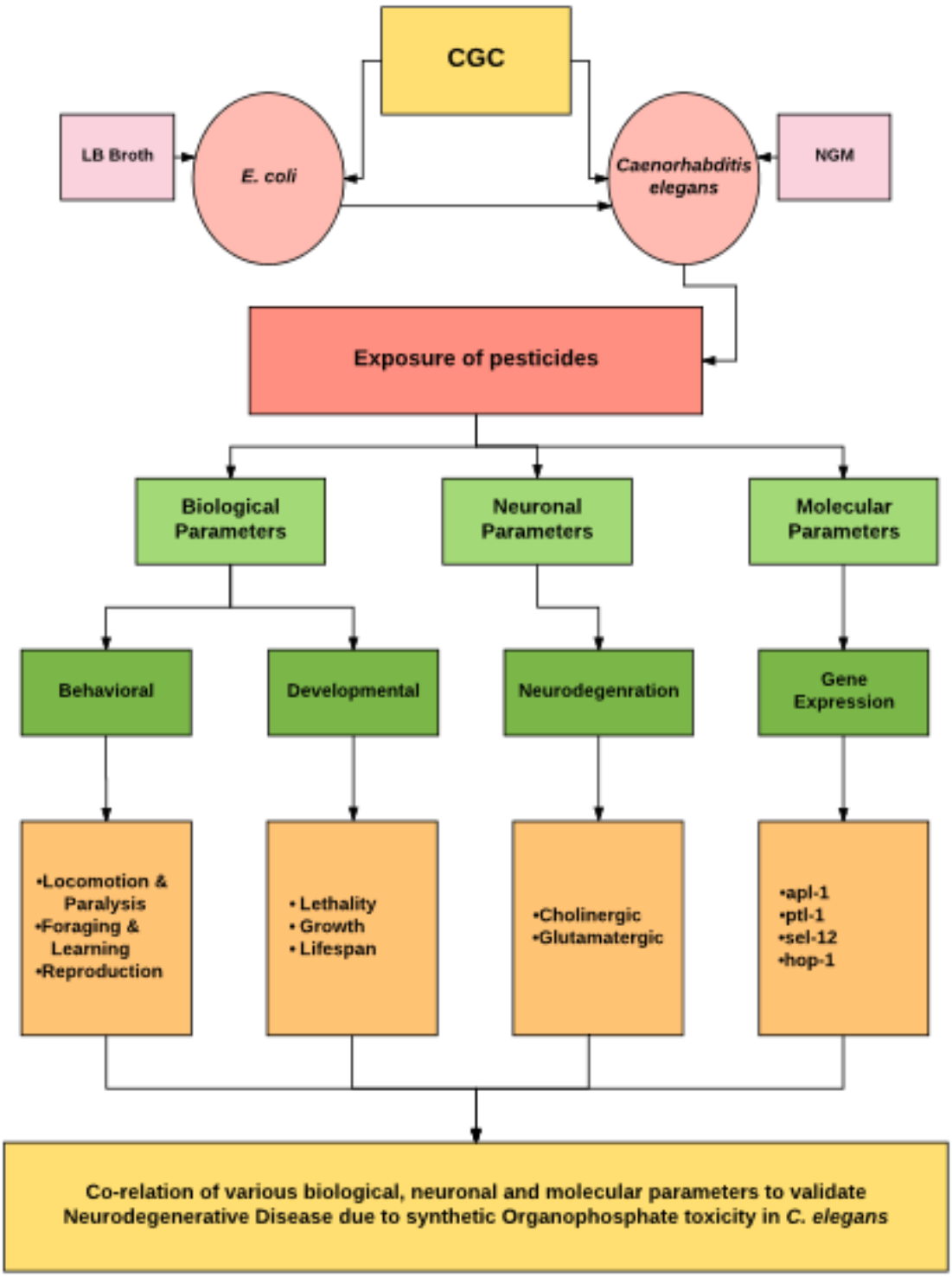
4.4 NEURONAL PARAMETER

Degeneration of cholinergic and glutamatergic neurons in pesticide treated worms will examined under dissecting fluorescence microscope. The exposed worms will be analyzed by immobilizing with 2% of sodium azide, pre-dropped on 2% agarose (Nass et al., 2001). Transgenic strain otls591 of unc-3::GFP fosmid reporter and otls388 for eat-4::GFP fosmid reporter may be used for the cholinergic and glutamatergic neurons respectively, for study of neurodegeneration (Kratsios et al., 2012). The result of neuron degeneration will be further analyzed by using various software like neurite tracer, genesis and Wis neuromath.

4.5 MOLECULAR PARAMETERS

Presence of genes such as apl-1, ptl-1, sel-12 and hop-1 after the pesticide exposure will be confirmed by two step Reverse Transcription Polymerase Chain Reaction (RT-PCR). First step includes the extraction of whole RNA content from the nematode worm. Second step includes cDNA synthesis from RNA. Further the DNA will be amplified by RT-PCR using gene specific primers and the amplified product will be analyzed by Agarose Gel Electrophoresis (Alexander *et al.,* 2014).

5.0 PLAN OF WORK



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